

Relationship of Cellulosomal and Noncellulosomal Xylanases of *Clostridium thermocellum* to Cellulose-Degrading Enzymes

ELY MORAG,^{1†} EDWARD A. BAYER,^{2*} AND RAPHAEL LAMED¹

Department of Biotechnology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv,¹ and
Department of Biophysics, The Weizmann Institute of Science, Rehovot 76100,² Israel

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Xylanase activity of *Clostridium thermocellum*, an anaerobic thermophilic cellulolytic bacterium, was characterized. The activity was localized both in the cellulosome (the principal multienzyme, cellulose-solubilizing protein complex) and in noncellulosomal fractions. Each of these fractions contained at least four major polypeptide bands which contributed to the xylanolytic activity. In both cases, pH and temperature optima, product pattern, and other features of the xylanase activity were almost identical. The main difference was in the average molecular weights of the respective polypeptides which appeared responsible for the activity. In the noncellulosomal fraction, xylanases with M_r s ranging from 30,000 to 65,000 were detected. Distinct from these were the cellulosomal xylanases, which exhibited much larger M_r s (up to 170,000). The cellulosome-associated xylanases corresponded to known cellulosomal subunits, some of which also exhibited endoglucanase activity, and others which coincided with subunits which appeared to express exoglucanase-like activity. In contrast, the noncellulosomal xylanases hydrolyzed xylan exclusively. β -Glucosidase and β -xylosidase activities were shown to be the action of different enzymes; both were associated exclusively with the cell and were not components of the cellulosome. Despite the lack of growth on and utilization of xylan or its degradation products, *C. thermocellum* produces a highly developed xylanolytic apparatus which is interlinked with its cellulase system.

In nature, potentially useful biomaterials often exist as complex multicomponent polymers. For example, the plant cell wall comprises an intricate structural array consisting mainly of cellulose, hemicellulose, and lignin. Although a variety of different bacteria and fungi can successfully degrade these substrates, only a little is known about the exact mechanism(s) of microbial degradation of such biomaterials.

Recent work in our laboratory (16, 17) has contributed to a better understanding of the efficient degradation of crystalline cellulose by the bacterium *Clostridium thermocellum*. The cellulosome, a high-molecular-weight multicomponent protein complex which exists in cell surface-bound and cell-free forms, has been shown to be responsible both for cellular adherence to cellulose and for the degradation of cellulose to cellobiose by the intact organism (5, 22). The strong adsorption to cellulose and the molecular organization to form a defined complex of the cellulosome have been proposed to generate efficient degradation of cellulose by the intact organism (4, 21). Indeed, the cellulosome was found to be responsible for the true cellulase activity (i.e., the capacity to fully solubilize cellulose) in *C. thermocellum* (20).

Previous publications (11, 26, 28) have also suggested the presence of xylanases in the extracellular growth medium of *C. thermocellum* despite the reported (10, 11) inability of this organism to grow on xylan as the sole source of carbon and energy. The definition of an enzyme as a xylanase is complicated by the commonly observed cross-specificity of xylanases and cellulases (13, 32). In one report (29), two different enzymes derived from *C. thermocellum* were described; one was shown to exhibit only carboxymethyl cellulase (CMCase) activity, whereas the other consisted of a combination of CMCase and xylanase activities. These

particular proteins, when purified, were found not to be associated into the high-molecular-weight complex (the cellulosome). It was therefore of interest to characterize in general terms the xylanase activities associated with the cellulosome and to compare their properties with those of noncellulosomal xylanases. In addition, we were interested in understanding the interaction of these proteins with xylan and the effect of xylan on their interaction with the preferred substrate, i.e., insoluble cellulose.

MATERIALS AND METHODS

Materials. Cellobiose, carboxymethyl cellulose (sodium salt, low viscosity), 4-methylumbelliferyl- β -D-cellobiopyranoside (MeUmb-cellobiose), 4-methylumbelliferyl- β -D-glucopyranoside, 4-methylumbelliferyl- β -D-xylopyranoside, *p*-nitrophenyl- β -D-glucopyranoside, and *p*-nitrophenyl- β -D-xylopyranoside were obtained from Sigma Chemical Co. (St. Louis, Mo.). Microcrystalline cellulose (Avicel) was purchased from E. Merck AG (Darmstadt, Federal Republic of Germany); xylose was a product of Fluka AG (Buchs, Switzerland). Xylobiose, xylotriose, and birch xylan (Roth, Karlsruhe, Federal Republic of Germany) were kindly provided by G. Zeikus, Department of Biochemistry, Michigan State University.

Xylan degradation products were prepared by subjecting a suspension containing 0.5% xylan to 48 h of incubation at 60°C in the presence of 5 μ g of purified cellulosome per ml. The reaction mixture was passed through a cellulose column to remove the residual cellulosome.

Organism, substrates, and culture conditions. Cultures of *C. thermocellum* YS were grown anaerobically at 60°C in serum bottles containing the previously described (18) medium which included the desired carbon source. Cells were grown on cellobiose for 8 to 9 h, according to predetermined growth curves. Cellulose-grown cells were cultivated for about 24 h (until the cellulose substrate was no longer visible

* Corresponding author.

† Formerly Ely Morgenstern.

Preparation of extracellular and cell-associated material. Extracellular (cell-free) material was obtained from the growth cultures by centrifuging the cells at $12,000 \times g$ for 30 min. The supernatant fluids were collected and concentrated 50-fold by ultrafiltration with an Amicon PM10 membrane (nominal M_r cutoff value, 10,000). Cell-associated material was prepared from whole-cell sonic extracts as follows. Cells, cultured in 200 ml of medium, were washed once with Tris buffer (pH 7.5) and resuspended in about 3 ml of the same buffer. The suspension was sonicated for 10 min in ice with a 70% duty cycle, using a tapered disruptor horn in a sonicator cell disruptor (model W-225 R; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). Particulate matter was removed by centrifugation ($30,000 \times g$) for 20 min.

Fractionation of extracellular xylanase. Extracellular constituents of cellulose-grown cells were fractionated either according to size or according to their interaction with cellulose or xylan. In the former case, 50-fold-concentrated extracellular material was subjected to gel filtration on a Sepharose 4B column (0.8 by 100 cm). The column was equilibrated and eluted with 50 mM Tris hydrochloride buffer (pH 7.5) containing 0.05% sodium azide. Fractionation on either cellulose or xylan was performed by bringing the unprocessed cell-free medium to 1.2% with the desired polymer. After a 1-h incubation at room temperature, the stirred suspension was centrifuged with a clinical centrifuge and the clarified supernatant was collected.

Purification of cellulosome. The cellulosome was purified from the spent cell-free growth medium of cellulose-grown cells of *C. thermocellum*. The procedure is based on a two-step process which includes affinity chromatography on cellulose and gel filtration on Sepharose 4B and has been described previously in detail (18).

Fractionation of xylan substrate. To prepare soluble and insoluble fractions of xylan, a suspension (0.5%) of commercial xylan was prepared and stirred for 1 h at room temperature. The mixture was centrifuged for 10 min at $17,300 \times g$; the supernatant, comprising the soluble fraction, was removed and saved; the pellet, comprising the insoluble fraction, was washed twice with 20 volumes of phosphate-buffered saline (PBS), collected, and saved.

Xylanase activity. Xylanase activity was assayed by incubating the desired enzyme preparation in the presence of 0.5% (wt/vol) xylan (total fraction) in citrate-phosphate buffer (50 mM K_2HPO_4 , 12.5 mM citric acid, pH 6.3) at 60°C (unless otherwise stated in the text). When a pH range was desired, citrate buffers were used between pH 2.9 and 7.4 and phosphate buffers were used between pH 6.3 and 8.3. There was essentially no difference in the results noted for the overlapping buffer region (pH 6.3 to 7.4). Appearance of reducing sugars was assayed by the dinitrosalicylic acid procedure (24).

In some cases, a radial enzyme assay was employed, particularly when assessing possible inhibition of xylanase activity by breakdown products and related sugars. The assay was done in xylan-embedded agar gels by the Congo red procedure (8).

Stability assay. Cellulosomal or noncellulosomal xylanases were treated with 0.2% sodium dodecyl sulfate (SDS), sample buffer, or sample buffer without mercaptoethanol. When desired, samples were heated to 70 or 100°C for 10 min. Xylanase activity was then determined as described above.

Analysis of reaction products. Xylan degradation products were qualitatively determined by thin-layer chromatography on DC-Plastikfolien Silica Gel 60 F₂₅₄ (Merck) with acetone-

ethyl acetate-acetic acid (2:1:1, vol/vol/vol). The plates were visualized by spraying with a 1:1 (vol/vol) mixture of 0.2% methanolic orcinol and 20% sulfuric acid; the plates were then heated.

CMCase activity. Endoglucanase activity was determined in vitro by the method of Miller et al. (24) with carboxymethyl cellulose as the substrate. The incubation was performed for 30 min at 60°C. A unit of CMCase activity was defined as the amount of enzyme which released 1 μ mol of reducing sugar (with glucose as the standard) per ml of sample per min under the conditions indicated.

β -Glucanase activity and β -xylosidase activity. β -Glucanase and β -xylosidase activities were estimated by measuring the release of *p*-nitrophenol from the appropriate substrate (*p*-nitrophenyl- β -D-glucopyranoside, *p*-nitrophenyl- β -D-xylopyranoside, or 4-methylumbelliferyl- β -D-xylopyranoside). Samples (5 μ l) were dissolved in 1 ml of citrate-phosphate buffer containing 10 mM of the corresponding substrate, and the reaction mixture was incubated for 30 min at 60°C. The reaction was terminated by adding 30 μ l of 2 M Na_2CO_3 , and the optical density of the liberated *p*-nitrophenol was measured at 405 nm. The release of 4-methylumbelliferone was measured at 347 nm by difference absorption spectrophotometry or by fluorometry (pH 10) at emission wavelengths of >435 nm (excitation, 335 to 400 nm).

Bacterial adhesion assay. A washed-cell suspension was brought to $A_{400} = 2$ with PBS. Increasing volumes of the cell suspension were brought to a total volume of 1 ml, and either 10 mg of xylan (insoluble fraction) or 10 mg of Avicel was added. The suspension was vortexed for 20 s and rocked gently at room temperature. After 20 min, the suspension was centrifuged with a clinical centrifuge (1,500 rpm) for 4 min. The supernatant fluids were collected, and the turbidity (A_{400}) was measured and compared with that of control tubes in which PBS was substituted for the cell suspension.

In some experiments, the effect of xylan on the adhesion of the bacterial cells to cellulose was evaluated by the addition of 2.5 mg of xylan (soluble fraction) per ml.

Cellulosome adhesion assay. The purified cellulosome was biotinylated by the method of Bayer and Wilchek (6). A solution of the biotinylated cellulosome was treated with increasing amounts of either microcrystalline cellulose or an insoluble xylan preparation. The suspension was rocked for 20 min at room temperature and centrifuged at $4,300 \times g$, and 1- μ l samples containing serial dilutions of the supernatant fluids were loaded onto nitrocellulose strips. The strips were quenched for 1 h with 2% bovine serum albumin in PBS, washed three times with PBS, and incubated for 30 min with solutions containing streptavidin complexes with biotinylated alkaline phosphatase (7). The strips were then rinsed again with PBS, and substrate solution (10 mg of naphthol AS-MX phosphate [sodium salt] and 30 mg of fast red TR salt dissolved in 100 ml of 0.1 M Tris hydrochloride buffer [pH 8.4]) was added. The reaction was terminated by washing the blots with distilled water. The amount of biotinylated enzyme remaining in each sample was determined either visually or by densitometric readings of the dot blots. The data were compared with a serially diluted biotinylated cellulosome standard.

Electrophoresis and in situ visualization of enzyme activities. SDS-polyacrylamide gel electrophoresis (PAGE) was performed in 6 or 10% gels essentially as described previously (15). The gels were stained for endoglucanase or xylanase activity in a modification of the cellulase assay described by Beguin (8). A suspension (0.1% final concen-

xylanase activity in a modification of the cellulase assay described by Beguin (8). A suspension (0.1% final concentration) of either carboxymethyl cellulose or soluble xylan was incorporated into the separating gel before the addition of ammonium persulfate and polymerization. Protein samples were applied to the gel after being heated for 10 min at the desired temperature (either 70 or 100°C) in the presence of sample buffer (3% SDS, 10% glycerol, 5% β -mercaptoethanol in 31 mM Tris hydrochloride [pH 6.8]).

Upon completion of electrophoresis, the gel was washed four times for 30 min in citrate-phosphate buffer (the first two washes contained 25% isopropanol) and the gel was then incubated in this buffer for 5 min at 60°C. The gel was introduced into a 0.1% solution of Congo red and gently rocked for several minutes. Excess dye was decanted, and the gel was washed with 1 M NaCl until excess stain was totally removed from the active band. After a final rinse in 5% acetic acid, the background turned dark blue, thus facilitating photographic documentation. In duplicate lanes, proteins were stained with Coomassie brilliant blue R250, destained, and photographed. In some cases, the gel was stained twice, once with Congo red and once with Coomassie brilliant blue. This enabled precise identification of activity with a given band.

Zymograms for exoglucanase activities. Because of the sensitivity of exoglucanase activity to boiling in SDS (see Results), protein samples were applied to gels after being heated for 5 min at 70°C in sample buffer. Upon completion of electrophoresis, the gels were washed twice for 30 min with citrate-phosphate buffer (pH 6.3) containing 25% isopropanol and twice for 30 min with the same buffer containing 1 mM MeUmb-cellobiose (without isopropanol). After being washed, the gels were incubated in the substrate-containing buffer for 20 min at 60°C. Positive bands were detected by fluorescence under UV illumination at 340 nm.

β -Glucosidase activity was similarly detected on gel overlays, except that samples (dissolved in buffered glycerol only) were not boiled before application to SDS-10% PAGE gels and 4-methylumbelliferyl- β -D-glucopyranoside was used as the substrate instead of MeUmb-cellobiose. β -Xylosidase activity was not stable even under these relatively mild conditions for SDS-PAGE.

HPLC. Gel permeation high-performance liquid chromatography (HPLC) was done with a model 303 HPLC system (Gilson Medical Electronics, Villiers le Bel, France) equipped with a spectrophotometer detector (model LC-75; The Perkin-Elmer Corp., Norwalk, Conn.). Cell-associated (sonic extract) material (200 μ l, ca. 2 mg) was applied to a prepacked Superose 12 gel filtration column (Pharmacia, Uppsala, Sweden). The column was developed at 0.4 ml/h with 0.1 M Tris hydrochloride (pH 7.5). Additional conditions conformed to the instructions of the manufacturer.

RESULTS

Xylanase activity in *C. thermocellum*. *C. thermocellum* was grown on either cellulose- or cellobiose-containing medium. The cultures were harvested in the late exponential phase of growth, and the respective fractions were examined for degradative activity on xylan. The cell-free xylanase activity derived from cellobiose-grown cells was about double that of the cell-associated activity (6.5 versus 3.1 μ mol of xylose per ml of broth per min). Similar but lower levels of xylanase activity were achieved for cellulose-grown cells (4.7 versus 1.4 μ mol per ml of broth per min for cell-free and cell-associated activity, respectively).

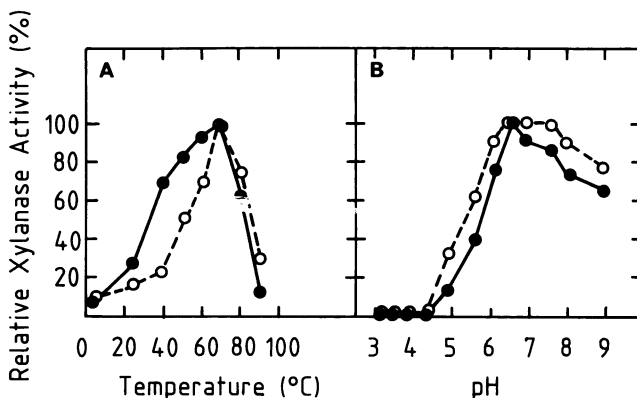


FIG. 1. Effect of temperature and pH on cellulosomal and non-cellulosomal xylanase activities. Samples of the purified cellulosome (●) and the noncellulosomal extract (○) were incubated with the substrate for 30 min at pH 6.3 at the indicated temperatures (A) or at 60°C at the designated pHs (B). In each experiment, the highest observed rate of hydrolysis was defined as 100% for the given preparation.

Fractionation of extracellular xylanase activity. It was of interest to determine the distribution of the observed xylanase activity of *C. thermocellum*. For this purpose, the extracellular constituents of cellulose-grown cells were fractionated either according to size or according to their interaction with cellulose or xylan. The cell-free growth medium was therefore subjected to gel filtration on Sepharose 4B, and the high- and low-molecular-weight fractions were pooled separately and concentrated and their respective xylanase activities were determined. The results showed that about 60% of the activity was associated with the high-molecular-weight fraction. In parallel, cellulose or xylan (1.2%) was introduced to the extracellular material, and after a 1-h incubation, the amount of xylanase activity adsorbed onto the corresponding matrix (after its removal by centrifugation) was determined. With cellulose, more than half of the xylanase activity was bound to the insoluble polymer. In contrast, with xylan, all the activity remained in the unbound state.

The association of xylanase activity with high-molecular-weight cellulose-bound fractions suggested that a major portion of this activity in *C. thermocellum* is contained in the cellulosome. We thus decided to further test this possibility.

Characteristics of xylanase activity in the cellulosome. The degradation of xylan by the purified cellulosome was analyzed, and a specific activity of 100 μ mol of xylose per min per mg was obtained. The activity reached a maximum within the pH range of 6 to 7.5, and the reaction exhibited a broad temperature optimum with a maximal activity of about 70°C (Fig. 1). The major products of the reaction included xylobiose (as the main reaction product), xylotriose, and various unidentified oligosaccharides (Fig. 2). Very low levels of xylose were also detected. After long periods of reaction (days), most of the xylotriose was converted to xylobiose (and xylose), which formed the major reaction product (data not shown).

The stability of the xylanase activity of the cellulosome after treatment with various agents was also investigated. Heating the cellulosome to 70°C for 10 min had little effect by itself on xylanase activity. Boiling the cellulosome, however, reduced the observed activity to about 15% of the original value. On the other hand, the combination of heating

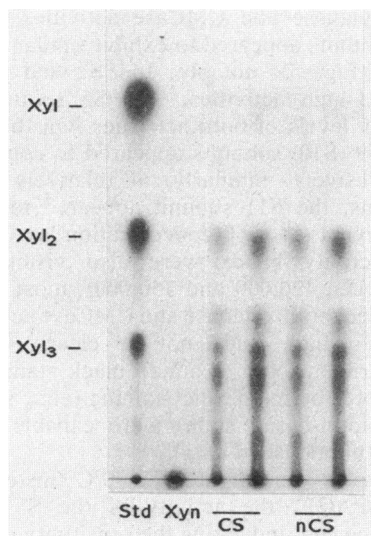


FIG. 2. Thin-layer chromatography of the major reaction products of xylanase activities in *C. thermocellum*. Cellulosomal (CS) or noncellulosomal (nCS) fractions were mixed with 0.5% xylan and incubated for 2 days at 60°C, and samples (0.3 and 1 μ l) of each were applied to the thin-layer chromatography plates. Untreated xylan (Xyn) and xylose oligomers (xylose [Xyl], xylobiose [Xyl₂], and xylotriase [Xyl₃]) were included as standards (Std).

at 70°C and the presence of SDS resulted in a significant reduction (70 to 80%) of the xylanase activity of the cellulosome.

In addition, the activity was sensitive to inhibition by certain divalent metal ions. Interaction with 10 mM concentrations of Cu²⁺ and Pb²⁺ inhibited most of the activity. Zn²⁺ and Co²⁺ were less effective inhibitors; Ca²⁺ and Mg²⁺ had no effect on the xylanase activity of the cellulosome. The order of sensitivity of cellulosome action to metal ions was Cu²⁺ > Pb²⁺ \gg Zn²⁺ > Co²⁺. The results are consistent with those of Tang et al. (29), who studied the action of a crude xylanase preparation using trinitrophenyl xylan as the substrate.

Since cellobiose, the end product of the cellulolytic process in *C. thermocellum*, is known to inhibit true cellulase activity in the cellulosome (14, 20), we also examined whether xylose, glucose, or cellobiose (2%, wt/vol) inhibited the xylanase activity, with negative results. Soluble xylan degradation products (0.25 mg/ml) also failed to significantly inhibit the activity.

Minute levels of a β -xylosidase-like activity were observed with either *p*-nitrophenyl- β -D-xylopyranoside or 4-methylumbelliferyl- β -D-xylopyranoside as the substrate (data not shown). However, the cellulosome failed to convert pure β -xylobiopyranoside to xylose. This tends to corroborate the very low levels of xylose observed as a degradation product with xylan as the substrate (Fig. 2) and indicates that the cellulosome essentially lacks strict β -xylosidase activity.

Interaction of the cellulosome with xylan. Despite the high levels of xylanase activity in the cellulosome, *C. thermocellum* YS failed to grow on either xylan or xylose as the major carbon and energy source. Since the cellulosome is an exocellular complex which, in addition to its enzyme apparatus, is responsible for the adhesion of the bacterium to the insoluble cellulose substrate, it was interesting to determine whether the cellulosome binds also to xylan. For this pur-

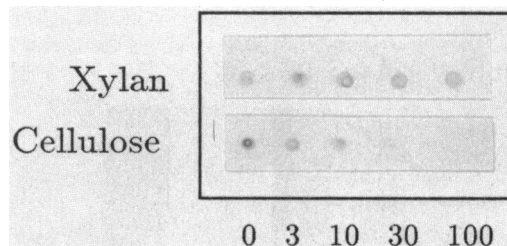


FIG. 3. Adsorption of the cellulosome to insoluble substrates. Various concentrations (milligrams per milliliter, as indicated) of insoluble xylan or cellulose were applied to a solution (1 mg/ml) of a biotinylated preparation of the purified cellulosome. The unadsorbed fraction was applied to dot blots, and the presence of residual (biotinylated) cellulosome was determined by a streptavidin-based staining procedure. Note that the cellulosome fails to adhere to xylan but adsorbs strongly to cellulose.

pose, a biotinylated preparation of the cellulosome was treated with incremented amounts of an insoluble xylan preparation, the polymer was pelleted, and the amount of biotinylated enzyme remaining in the supernatant fluids was determined. The results were compared with those of a positive control experiment in which cellulose was used as the adsorbent. With xylan, essentially all the cellulosome was found in the supernatant fraction, indicating a lack of binding to the substrate (Fig. 3). In contrast, the cellulosome was completely adsorbed to relatively low amounts of cellulose. Moreover, the addition of large amounts (10-fold) of xylan (soluble fraction) to the cellulosome-containing medium failed to interfere with the adsorption of the cellulosome to cellulose.

Noncellulosomal xylanases. In many respects, the characteristics of the noncellulosomal xylanase activity were very similar to those exhibited by the cellulosome. The pH maximum of the noncellulosomal xylanases was found to be identical to that of the cellulosome, although a much broader pH optimum was evident in the former (Fig. 1B). Likewise, the temperature maximum was identical in the two xylanase forms in *C. thermocellum*, except that a somewhat sharper temperature optimum was observed for the noncellulosomal xylanases (Fig. 1A).

The reaction products were indistinguishable for cellulosomal and noncellulosomal activities; xylobiose is the predominant product (Fig. 2). In both cases, complete depolymerization was evident from thin-layer chromatographic analysis after long incubation periods. Interestingly, the inhibitory effect of divalent metal ions on the noncellulosomal xylanase activity was essentially identical to that on the cellulosomal activity, i.e., Cu²⁺ > Pb²⁺ \gg Zn²⁺ > Co²⁺.

Comparative polypeptide pattern of cellulosomal and non-cellulosomal xylanases. Despite the striking similarities in the above-described characteristics of the cellulosomal and non-cellulosomal xylanase activities, their respective polypeptide patterns were markedly different (Fig. 4). The noncellulosomal polypeptides ranged from *M_r* ~27,000 to 70,000, far below the average molecular weights of (and migrating in positions inconsistent with) the cellulosomal subunits, which ranged from *M_r* 48,000 to 210,000.

Even more pronounced, however, was the difference in the regenerated xylanase activities after SDS-PAGE separation. In the cellulosome-associated fraction, several major xylanase activities could be detected, corresponding to molecular weights of 170,000, 84,000, 67,000, and 54,000 (i.e., S2, S7, S9 and/or S10, and S13, respectively). Two to three minor activities, e.g., S3, S5, and S11 (molecular

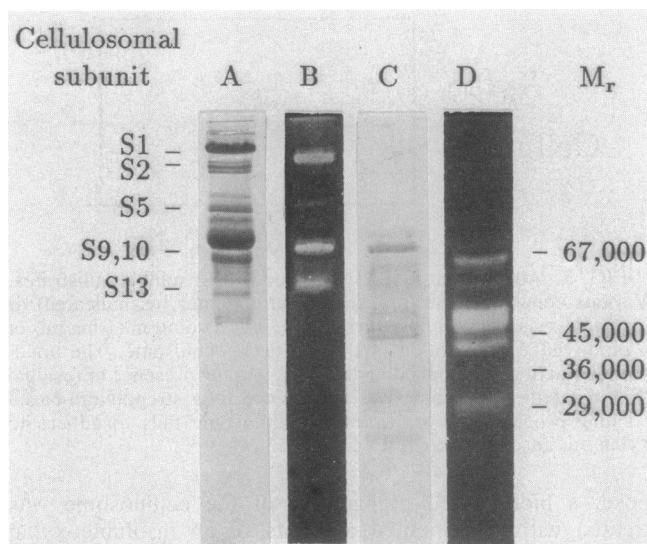


FIG. 4. Xylanolytic activity profile of SDS-PAGE-separated celulosomal and noncelulosomal components. The purified celulosome (lanes A and B) and noncelulosomal (lanes C and D) extracts were boiled for 10 min and applied to SDS-10% PAGE gels. Lanes were either stained with Coomassie brilliant blue (lanes A and C) or examined for xylanase activity (lanes B and D) by the Congo red procedure.

weights of 150,000, 98,000, and 60,000, respectively), could also be discerned. Regarding the association of enzymatic activities with the S9 and S10 subunits, these components exhibit similar M_r s (21) and are difficult to resolve under the SDS-PAGE conditions used in this study.

In the noncelulosomal fraction, none of the major xylanase activities were coincident with those of the celulosome. The major noncelulosomal xylanase activities exhibited M_r s of 65,000, 47,000, 45,000, 40,000, and 30,000.

Xylanase and endoglucanase activities of celulosomal subunits. When parallel samples of the celulosome (treated at 100°C in sample buffer) were subjected to SDS-PAGE and

tested for xylanase and CMCase activities, some of the separated subunits appeared to exhibit similar levels of both specificities (Fig. 5); notably, the S2 and S13 subunits showed very high activities. The S5 subunit expressed relatively low levels of both activities. On the other hand, the S7 and S9 (S10) subunits appeared to contain xylanase activity exclusively. Similarly, at relatively high sample concentrations, the S11 subunit appeared to exhibit only xylanase activity. At such concentration levels, a series of additional activity bands were also visible (molecular weights between 120,000 and 150,000), most of which appeared to reflect both xylanase and CMCase activities. Many of the latter bands could not be clearly discerned by Coomassie brilliant blue or amido black staining, although other types of protein-specific staining (e.g., silver staining and avidin-biotin-based stain) were capable of resolving these bands (unpublished data).

If the samples were treated at 70°C (instead of 100°C) before SDS-PAGE, the activity of the S3 subunit was markedly enhanced, indicating the sensitivity of this subunit to boiling in the presence of SDS. These conditions (incubation at 70°C in SDS) were used because they are the mildest by which complete denaturation of the celulosome is effected. Other bands may well be sensitive to treatment at 70°C, and lack of observed enzyme activity does not necessarily mean that a given band does not contribute the respective activity to the celulosome complex in situ. On the other hand, using CMCase zymograms, we could not examine celulosome samples treated at 70°C, since the subunits retained high levels of activity during electrophoresis, thereby causing a smearing effect resulting in poorly resolved bands.

Exoglucanase-like activity of celulosomal subunits. In the past, MeUmb-cellobiose has been used as an indication of exoglucanase (specifically cellobiohydrolase) activity (9, 27), although there is serious doubt as to the validity of its use for this purpose. With MeUmb-cellobiose as the substrate (Fig. 6), the highest activity levels were observed associated with the S3, S5, and S9 (S10) subunits. Lower levels of activity were detected in the S4 and S6 or S7 subunits. In this work,

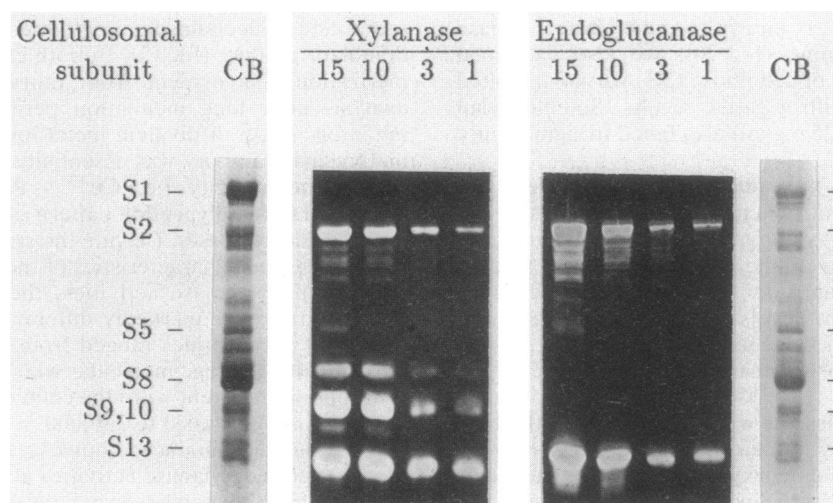


FIG. 5. Comparison of xylanase versus endoglucanase activities in the celulosome. Various amounts (micrograms, as indicated) of the purified celulosome were heated in sample buffer to 100°C and applied to SDS-6% PAGE gels. After electrophoresis, the gels were examined for xylanase or CMCase activity by the Congo red procedure. Coomassie brilliant blue-stained celulosome samples (lanes CB) are presented for reference.

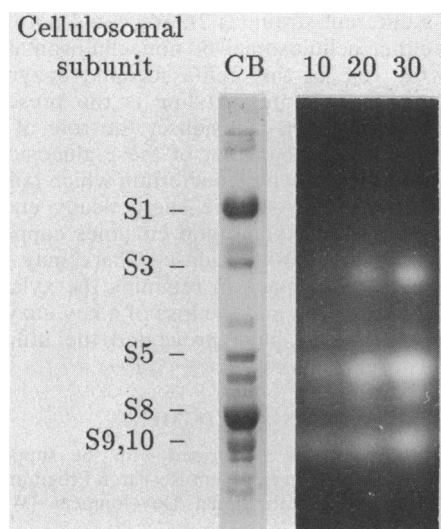


FIG. 6. Distribution of exoglucanase-like activity in the cellulosome. Various amounts (micrograms, as indicated) of the purified cellulosome were heated in sample buffer at 70°C and subjected to SDS-PAGE (6% separating gels). The gels were then incubated with MeUmb-cellobiose as described in the text. A Coomassie brilliant blue-stained cellulosome sample (CB) is provided as a reference.

we do not intend to argue the legitimacy of MeUmb-cellobiose as a strict exoglucanase substrate; in this context, we refer to the activity on this artificial substrate as exoglucanase-like activity.

Disposition of β -glucosidase and β -xylosidase activities in *C. thermocellum*. β -Glucosidase and β -xylosidase activities were found exclusively in cell-associated extracts. Specific activities of 0.095 $\mu\text{mol/min}$ per mg of protein (0.020 $\mu\text{mol/min}$ per ml of broth) for β -xylosidase and 0.25 $\mu\text{mol/min}$ per mg of protein (0.062 $\mu\text{mol/min}$ per ml of broth) for β -glucosidase were obtained. Both activities were essentially absent from cell-free (cellulosomal and noncellulosomal) fractions. It was therefore of interest to ascertain whether the activities simply reflected cross-specificities of a single enzyme or whether they represented the actions of individual entities.

Gel permeation HPLC of the whole-cell extract of *C. thermocellum* revealed that the respective activities were confined to separate peaks (Fig. 7), one representing β -glucosidase activity (M_r , ~55,000) and a second which exhibited β -xylosidase activity (M_r , ~200,000). SDS-PAGE of the partially purified β -glucosidase combined with the relevant zymogram (with 4-methylumbelliferyl- β -D-glucopyranoside) revealed a single band (M_r , ~50,000). Similar electrophoretic analysis of the β -xylosidase was inapplicable, since the enzyme activity was abolished in the presence of SDS.

DISCUSSION

The purpose of this study was to characterize the xylanase activity exhibited by the *C. thermocellum* YS system and to evaluate whether such activity represents simply a subsidiary activity of the cellulase system or an independent set of enzymes devoted to the degradation of hemicellulose. In addition, information was lacking regarding exocellulase activity in the cellulosome and regarding the occurrence and location of β -glucosidase, the role of which may be somewhat ambiguous in view of the previously reported alternative phosphorylation-associated cleavage of cellobiose (3).

Although *C. thermocellum* neither utilizes xylan nor ad-

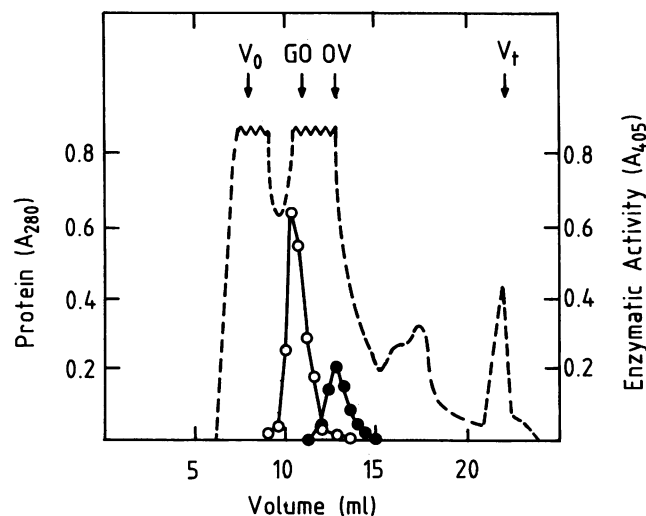


FIG. 7. Gel permeation HPLC of cell-associated β -glucosidase and β -xylosidase activities. Whole-cell sonic extract (200 μl) was separated by HPLC on a Superose 12 column. The fractions were analyzed for protein content (---) and β -glucosidase (●) and β -xylosidase (○) activities. The positions of the void volume (V_0), glucose oxidase (GO; molecular weight, 160,000), ovalbumin (OV; molecular weight, 45,000), and the total volume (V_t) of the column are given.

heres to the insoluble polymer, it is quite likely that the degradation of hemicellulose would enhance the ability of the organism to reach the cellulosic component(s) of the plant cell wall substrate. Furthermore, natural degradation of plant material occurs in most cases by mixed cultures in which the cellulolytic bacterium may contribute carbon sources to the immediate environment by virtue of its hydrolytic enzymes, benefitting, at the same time, from nutrients and/or the removal of toxicants by the auxiliary bacteria (19, 25). In this context, *C. thermocellum* may provide the polymer-degrading link to satellite bacterial strains which are dependent on the rapid dispersal of low-molecular-weight saccharides for growth.

It thus may not be surprising that at least eight xylanolytic polypeptides were found in this work to be secreted in *C. thermocellum* YS, many of which were identified to be associated with the cellulosome complex. The cellulosome, which exhibits high affinity to cellulose (20, 21), appears to be a targeting tool to bring these xylanase enzymes to the surface of the insoluble hemicellulose-containing cellulosic substrate (i.e., plant cell tissue); interaction of either the intact bacterium or the purified cellulosome with soluble or insoluble xylans could not be detected. This is somewhat surprising if we consider the potential for cooperative binding properties among the many xylanases within the cellulosome complex.

While the study of the free (noncellulosomal) xylanases may be relatively simple, the study of individual cellulosome components is rendered very difficult by the extreme stability of the complex (16, 22). Nevertheless, the capacity of the xylanase components (or at least a portion thereof) to undergo functional regeneration after separation by SDS-PAGE has allowed their identification in this work. In a complementary approach, Grepinet et al. (12) have cloned and expressed one of the xylanase genes (*xynZ*) in *Escherichia coli*. The xylanolytic protein did not exhibit CMCase activity but was shown to correspond to one of the subunits

(S5) of the cellulosome. In another recent work (23), three distinctly different xylanase genes from *C. thermocellum* have also been cloned and expressed in *E. coli*, two of which were considered atypical xylanases of limited activity and a third gene product which was isolated and shown to extensively hydrolyze xylan. In addition, Pape et al. (S. Pape, F. Mayer, and J. Wiegel, Abstr. 1st Annu. Meet. Am. Soc. Microbiol. Biotechnol. 1988, O-15) presented evidence using a different strain (JW20) of *C. thermocellum* which suggested that the cellulase system indeed contains other noncellulolytic activities (including xylanases). In this regard, Wiegel and Dykstra (31) reported low levels of adsorption of *C. thermocellum* to hemicelluloses extracted from steam-exploded wood. We could not detect any adhesion of the YS strain to the insoluble fraction of commercial birch xylan.

The general properties of the cellulosome-associated and noncellulosomal xylanase activities can be compared among themselves and in relation to the xylanase purified from *C. thermocellum* by Tang et al. (29). In doing so, we could not find significant differences in sensitivity to bivalent metal ions and pH and temperature optima, except for the relatively high molecular weights of the cellulosome-associated xylanases relative to those of the noncellulosomal system.

The multiplicity of xylanases observed in *C. thermocellum* may be caused by a variety of factors. The most important, perhaps, is the heterogeneous nature of the substrate, in particular the sugar substituents of the linear β -1,4-poly-D-xylopyranoside chain (e.g., arabinose, glucose, mannose, galactose, etc.), which constitute about 40% of most commercial xylans (30) and up to 80% of hemicellulose (32). Additional heterogeneity is caused by significant levels of acetylation of most xylans, in particular in hardwoods. The complex nature of the substrate may thus require an equally complex set of enzymes for degradation. On the other hand, post-translational modifications of the enzymes, such as glycosylation, may also contribute to the variety of individual xylanase polypeptides found in this organism.

One of the unexpected results of this study was that the purified cellulosome exhibits higher levels of xylanolytic activity than endoglucanase activity. This was also reflected in the number of distinct cellulosome subunits which express the respective activities after SDS-PAGE. In this regard, some of the cellulosomal subunits, notably S2 and S13, exhibited both xylanase and endoglucanase activities, whereas others, such as S7 and S9 (S10), appeared to express xylanase activity alone. In the noncellulosomal fraction, all the observed xylanases failed to exhibit detectable endoglucanase activity.

Nevertheless, the apparent predominance of xylanase activity over endoglucanase activity should be treated with reservation. In this context, xylan is an enzymatically more practical substrate than cellulose; the assay methods described here for xylanase activity are reasonably reflective of the hydrolytic capacity of the relevant enzymes for their substrate. On the other hand, cellulose degradation is an elusive commodity, and endoglucanase activity is not necessarily an accurate or even representative (and certainly not the sole) measure of cellulolytic activity per se. For this reason, we also attempted to characterize exoglucanase-like activity in the cellulosome. In this case, the pattern of label (with MeUmb-cellobiose as the substrate) showed a distinct labeling pattern, but definitive conclusions regarding true exoglucanase activity await more extensive end product analysis of separated (or cloned) subunits.

The presence of a cell-associated β -glucosidase in *C. thermocellum* YS corroborates previous findings of this

enzyme in a different strain (1, 2). We could not trace such activity in either cellulosomal or noncellulosomal preparations, and the role of this cell-associated enzyme is not entirely clear. Even more puzzling is the presence of a distinct cell-associated β -xylosidase, the role of which is even more ambiguous than that of the β -glucosidase. It is difficult to comprehend why a bacterium which fails to grow on (or utilize) xylose would be energetically encumbered with a complex set of xylanolytic enzymes capped with a seemingly superfluous β -xylosidase. There may still be a cryptic ecological advantage in retaining the xylanase system in *C. thermocellum*, and the loss of a key enzyme (e.g., xylose isomerase) may have prevented the utilization of xylose in this organism.

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